Winter School on

'RECENT ADVANCES IN DIAGNOSIS AND MANAGEMENT OF DISEASES IN MARICULTURE'

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Course Manual

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BIOTECHNOLOGY IN AQUACULTURE HEALTH MANAGEMENT

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Any technique that uses living organisms or substances from these organisms to make or modify a product or to improve plant or animal or to develop microorganism for specific uses, can be termed biotechnology. Biotechnology involves microbes or cells derived from plants and animals for efficient production of useful products for mankind. The use of microbes for production of alcohol and penicillin, to name a few, is well known. However, in the last 10-15 years, several novel organisms, yielding useful products have been created by genetic engineering techniques. Today, there is a wide application of biotechnology in human medicine and animal husbandry in the area of diagnosis, prophylaxis and therapy.

Aquaculture industry has undergone a sea change world over in the last decade. The important needs of the industry are production of fast growing disease resistant varieties, development of cheap and effective vaccines, disease diagnostic methods, cell lines and probiotics. Efforts on these lines have already begun and results are highly promising. Some of the biotechnologies employed in aquaculture health management are hybridoma technology, cell culture, subunit vaccine, diagnostics, bioremediation, probiotics and transgenic fish.

Hybridoma Technology

Hybridoma technology for production of monoclonal antibodies (MAbs) has contributed significantly to aquaculture. Monoclonal antibodies are being employed in disease diagnosis, pathogen classification, epidemiological analysis and development of vaccines.

Importance of Monoclonal Antibodies

Conventionally, antiserum (polyclonal antibodies) is prepared in rabbits for use in diagnostics, serotyping and vaccine development. Rabbits are immunized with a purified antigen with an adjuvant in 2 to 3 doses and the animal is killed and blood collected at the end of 30-40 days. The serum collected after blood clotting contains specific antibodies to the antigen injected. Rabbit antiserum contains many different types of antibodies derived from several plasma cell clones (polyclonal) that are specific to different epitopes. Even in a hyperimmune animal, seldom are more than one-tenth of the circulating antibodies specific for one antigen. The use of these mixed populations of antibodies creates a variety of different problems in immunochemical techniques such as background reaction and false positives. Besides, antiserum is not sensitive for detection of minute difference between antigens. In addition, availability of rabbit antiserum in limited quantities is also a serious drawback. Therefore, the preparation of large quantity homogenous antibodies with a defined specificity was a long-standing goal of immunochemical research. This goal of producing monoclonal antibodies was achieved with the development of the technology of hybridoma production.

Production of Monoclonal Antibodies

In animals, antibodies are synthesised primarily by plasma cells, a type of terminally differentiated B lymphocytes. Because plasma cells cannot be grown in tissue culture, they cannot be used as an *in vitro* source of antibodies. Kohler and Milstein (1975) developed a technique that allows the growth of clonal populations of cells secreting antibodies with a defined specificity. In this technique, an antibody-secreting cell, isolated from an immunised animal, is fused with a myeloma cell, a type of B-cell tumor. These hybrid cells or hybridomas can be cloned and maintained *in vitro* and will continue to secrete antibodies with a defined specificity. Antibodies that are produced by hybridomas are known as monoclonal antibodies (MAbs).

The usefulness of monoclonal antibodies stems from three characteristics --their specificity of binding, their homogeneity, and their ability to be produced in unlimited quantities (Table 1). The production of monoclonal antibodies allows the isolation of reagents with a unique, chosen specificity. Because, all the antibodies produced by descendants of one hybridoma cell are identical, monoclonal antibodies are powerful reagents for testing for the presence of a desired epitope. Hybridoma cell lines also provide an unlimited supply of antibodies. Therefore, production of immortal hybridoma will be much cheaper in the long run compared to production of rabbit antisera. In addition, one unique advantage of hybridoma production is that impure antigens can be used in immunisation to produce specific antibodies. Because hybridomas are single-cell clone prior to use, monospecific antibodies can be produced after immunisations with complex mixture of antigens.

Hybridoma secreting monoclonal antibodies specific for a wide range of epitopes have been prepared. Any substance that can elicit a humoral response can be used to prepare monoclonal antibodies. Their specificities range from proteins to carbohydrates to nucleic acids. However, production of monoclonal antibodies are often more time-consuming and costly than polyclonal antibodies. In theory, either as single antibody preparation or as pools, monoclonal antibodies can be used for all the tasks that require or benefit from the use of polyclonal antibodies. In practice, however, producing exactly the right set of monoclonal antibodies is often a difficult and laborious job.

Table 1 Comparison between antiserum (polyclonal) and monoclonal antibodies

Criteria	Antiserum	Monoclonal antibody
Antigenic determinant/ epitope recognition	Several	Single
Specificity	Variable with animal and breed. Partial cross-reactions with common determinants, seldom too specific	Standard, highly specific Unexpected cross reactions may occur May be too specific for requirement
Affinity	Variable with breed	May be selected during cloning
Yield of useful antibody	Up to 1 mg/ml	Up to 100μg/ml in tissue culture Up to 20mg/ml in ascitic fluid
Contaminating immunoglobulins	Up to 100%	None in cell culture, 10% in ascitic fluid
Purity of antigen required	Pure antigen is required	Some degree of antigen purification desirable but not essential

Approximate minimum cost (Rs.)	Usually below 5000 for limited quantity of antiserum	Capital cost – 10 lakhs. Running costs – 2 lakhs for unlimited quantity of highly specific antibody.

Application of Monoclonal Antibodies in Aquaculture

Today, monoclonal antibodies to several viral and bacterial pathogens of fish and shellfish are available in the market. It has been possible to develop rapid, simple, cheap, specific and sensitive MAb based immunodiagnostic kits for several microbial pathogens. Principle and methods of antibody based diagnostics have been dealt in detail in Chapter 12. MAb based diagnostic kits such as ELISA and Immunodot have even been simplified to the field level for use by farmers. Furthermore, detection of minute serological difference among bacterial and viral variants of fish and shellfish is possible by MAb based epitope analysis. This has helped immensely in serological and epidemiological studies. Monoclonal antibodies have also been extensively used in detection of epitopes involved in pathogenesis for development of subunit vaccines.

In India, in the authors laboratory MAbs to A. hydrophila, EUS fungus *Aphanomyces invadans* and white spot virus of shrimp have been produced and being used in diagnosis.

Fish Cell Lines

Development of fish cell lines has several applications in aquaculture such as in study and diagnosis of fish viruses, production of viral vaccines and aquatic toxicology. To date, there are about 60-70 cell lines derived from carp, catfish, salmonids, perches, mullets and snakeheads.

Cell lines are transformed cells that are adapted well to grow and multiply outside the host in *in vitro* culture systems. Cells lines can be developed from fast growing tissues of young fish. There are two methods of developing cell lines (1) explant method, (2) enzymatic method. Cell lines are grown in sterile plastic or glass containers containing appropriate medium having nutrients, salts, antibiotics, buffers, serum etc. Minimum essential medium (MEM) is the common medium employed for fish cell line. Cell lines are developed, characterised and deposited in national and international repositories, which can be procured for use in research and industry

Importance of Fish Cell Lines

Fish virology

Fish cell lines have an important role in the expanding aquaculture industry of the world. Undoubtedly, the most widely employed application of fish cell cultures is for the study of fish viruses. Indeed, the primary impetus, for the development of many of the continuous fish cell lines was to provide the means for isolating and identifying viruses that are the causative agents of epizootics of commercially important species. Therefore, most fish cell cultures have originated in North America and Europe where aquaculture developed on scientific basis earlier than in Asia. For example, at least 18 continuous cell lines exist from salmonid fishes such as trout and salmon, 9 lines from carp and 5 from catfish. Some of the most widely used fish cell lines are listed in Table 2.

Table 2 Examples of widely used fish cell lines

Cell line designation	Cell morphology	Fish
RTG-2	Fibroblast	*Salmo gairdneri* (Rainbow trout)
CHSE-214	Epithelioid	*Oncorhynchus* (Chinook salmon)
BF-2	Fibroblast	*Lepomis macrochirus* (Bulegill)
CAR	Fibroblast	*Carassius auratus* (Gold fish)
EPC	Epithelioid	*Cyprinus carpio* (Carp)
FHM	Epithelioid	*Pimephales promelas* (Fathead minnow)
BB	Epithelioid	*Ictalurus punctatus* (Brown bullhead)

In addition to the cell lines listed in the table 2, it should be noted that many new continuous fish cell lines are constantly being developed as a result of intensive efforts in several parts of the world, to provide cell cultures from local species utilized in aquaculture. As the scale and intensity of aquaculture continues to increase worldwide with consequent viral disease problems, the number of new cell lines will continue to increase. Examples of recently developed cell lines include those from common carp, loach, milkfish, eel, zebra fish, *Channa striatus*, gold fish, catfish and seabass. Although these cell lines were developed in Asia and Europe they should also find application in other parts of the world where these and/or related species of fish are reared. Besides diagnosis, cell lines are important in the much required national and international quarantine and certification programme for procuring virus free fish stock.

Fish cell lines are essential in diagnosis and identification of virus. Further, cell lines are essential for production of viral vaccines.

Cytogenetics

Fish cell cultures have found wide spread application for determination of karyotypes and other aspects of cytogenetics. Cultures derived from various organs, embryonic tissues, and other leucocytes have been used for the study of chromosomal polymorphism, speciation and evolution.

***In Vitro* Models**

Fish cell cultures also provide *in vitro* systems for studying various cellular and physiological processes. For example, organ cultures of pituitary glands were derived from tilapia, eel and rainbow trout for use in studying the production of growth hormone prolactin. Primary monolayer culture from the pituitary of tilapia, rainbow trout and dwarf bream have also been used for the same purpose. Also organ cultures of carp pituitary cells which produce gonadotropin have been developed. These cells should provide a useful tool for investigating several aspects of the hypothalamo-hypophyseal system of cyprinid fish.

In recent years fish cell lines are being used as in vitro models to evaluate the toxicity of pollutants. This is a better alternative to use of live fish in toxicity studies. Besides cell line *in vitro* models provide specific information in toxicology at cellular level.

State of Art of Fish Cell Lines in the Indian Subcontinent

Over the last decade there is tremendous growth in culture and breeding of carps, murrels, mullets and ornamental fishes in India. Besides, an increased activity in transport and exchange of seed and brood stock from one place to another and even across international border has been witnessed. Under such circumstances, incidence of disease of viral etiology will be common. There is little work on development of fish cell lines from Indian subcontinent perhaps because of lack of facilities and trained personnel. Since cell cultures derived from the same species or a species closely related to that in which the disease occurs would be the most sensitive for virus isolation, cell lines derived from local species should be given high priority. Though there are cyprinid derived cell lines such as EPC and CAR, development of cell lines from the widely cultivated Indian major carps should be given top priority. At present cell line from Mrigal is available in India. Published information from India is also available on the standardisation of explant method to develop cell lines from the heart tissue of Indian major carp and gonads of *Clarias batrachus*. There is need to initiate cell lines of the cultivable species, particularly for viral disease diagnosis, screening and certification of fish for import and export.

Subunit Vaccine

Development of subunit vaccines against viral and bacterial diseases is the need of the hour for use in intensive aquaculture. Subunit vaccines are effective, safe, cheap and can be produced in large quantities. The major problems with the commonly employed whole cell vaccines are

1. protective antigens are often destroyed during inactivation,
2. unwanted side effects and antigenic competition may occur as the result of complex mixture of microbial products present,
3. correct type of immune response (humoral, local or CMI) may not be always induced. Thus the vaccine produced by this approach are often far from perfect and hence need for alternatives such as subunit vaccines. The subunits are useful where the pathogen is difficult to grow, where microbial products are in very small quantity, or in development of vaccines for multicellular parasites. Once developed, subunits will become the cheapest, safest vaccine in large quantity.

Diagnosis by Gene Probes

Disease diagnosis by gene probes is the most sensitive and specific method. For large scale application in field there is a need of specific nucleic acid probes against several pathogens infecting fish and shellfish. Genetic engineering methods are being used for large scale preparation of these probes. DNA based techniques such as DNA hybridization and PCR have revolutionised diagnostics and disease management

Transgenic Fish

Production of fast growing and disease resistant varieties of fish is one of the important requirements in aquaculture. Researchers have successfully produced fast growing transgenic salmon, trout and common carp with incorporation of growth hormone gene. Attempts have been made to produce fast growing transgenic *Clarias sp* in India. On similar lines cold and disease resistant trouts and salmon have been produced by transgenesis. The method in simple involves identification of genes responsible for disease resistance, their isolation, and preparation of gene construct. The gene

constructed is transferred to fish eggs through microinjection and checked for its incorporation and successful expression in adults.

Probiotics and Bioremediation

Probiotics and bioaugmentation are being increasingly used in aquaculture. In bio-augmentation useful microbes are inoculated and enriched to improve pond bottom for decreasing accumulated waste. Use of biomats for improving water quality for reducing NH_3 level is in practice in fish and shellfish aquaculture. Useful microbes are immobilised on inert or biodegradable material for use in fish and shrimp ponds. Probiotic technique encourages beneficial bacterial species in the host or in water while suppressing the other, helping in disease prevention and growth promotion.